Gastrointestinal cell proliferation and crypt fission are separate but complementary means of increasing tissue mass following infusion of epidermal growth factor in rats

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Abstract

Background and aims—Epidermal growth factor (EGF) is a potent mitogen for the gastrointestinal tract and also influences the number of new crypts formed by crypt fission. The time course of these events and possible linkage between these two complementary mechanisms is however poorly understood. We therefore examined the temporal relationship of proliferation and fission in rats treated with EGF.

Methods—Osmotic minipumps were implanted subcutaneously into male Wistar rats to infuse EGF continuously (60 µg/rat/day) for periods of 1–14 days. Proliferation and crypt branching were quantified following vincristine induced metaphase arrest and morphometric assessment of microdissected tissue.

Results—In the small intestine, EGF significantly increased epithelial cell proliferation and crypt and villus area after 24 hours of EGF, although maximal effects were only reached following six days of infusion. EGF also resulted in an approximate 30% reduction in crypt fission in the small bowel. In the colon, EGF caused a twofold increase in epithelial cell proliferation one day after infusion, from 15.3 (2.3) to 29.6 (3.5) metaphases per crypt (p<0.01). Maximal effects were seen in rats receiving EGF for seven days. For all time points, colonic crypt size increased in response to EGF. The amount of branching increased following one day of infusion with EGF (from 15.3 (1.9) to 32.4 (5.5)%; p<0.001) but was significantly lower (approximately 25% of control values) following longer periods of infusion. Crypt fission did not correlate with crypt area.

Conclusion—EGF has profound effects on cell proliferation and also altered crypt fission, with its actions on crypt fission most pronounced in the colon where it first increased and then decreased fission. EGF can thus be a potent stimulus for crypt fission during short term infusion and may reduce the number of branched crypts present in a resting or quiescent stage. Growth factors can alter cell mass by two separate but linked mechanisms, namely altered cell production and crypt fission.

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The gastrointestinal tract is one of the most rapidly proliferating organs in the body, second only to the haematopoietic system. The mechanisms underlying this process are however poorly understood. Although there has been a great deal of research examining changes in proliferation in response to growth factors, it has been recently appreciated that an additional mechanism involving the production of new crypts is also important in mediating growth. New crypts are produced by a process termed crypt fission.[1] In this system, a founder crypt divides into two or more daughter crypts, thereby allowing clonal expansion. Crypt branching, which can be detected morphometricaly, is the early visible stage of the process. The controlling mechanisms underlying crypt fission are however poorly defined. We therefore performed a detailed temporal study to examine the effect of the potent growth factor epidermal growth factor (EGF)[2 3] on crypt proliferation, compartment size, and fission in the rat gut to determine whether proliferation is inextricably linked to crypt fission or is an independent but complementary process.

Methods

Recombinant EGF was produced by the Center for Genetic Engineering and Biotechnology, Havana, Cuba, from expression of a synthetic gene in *Escherichia coli* and was a mixture of EGF_{1-51} and EGF_{1-52}, was as biological active as full length EGF_{1-53},[4] and was 99% pure, as determined by high pressure liquid chromatography.

Male 200 g Wistar rats were anaesthetised with halothane and a small incision made between the shoulders in the skin of the dorsal neck. A pair of scissors was then used to make a tunnel under the skin and a 2 ml, 14 day Alzet osmotic minipump (model 2ml2; Charles River UK Ltd, Kent, UK) was inserted and the skin closed with a 4-0 wire suture. The pumps were filled with EGF or saline, and had an output of 5.25 µl/hour (126 µl/day) and thus a 0.48 µg/ml solution gave a daily output of 60 µg of
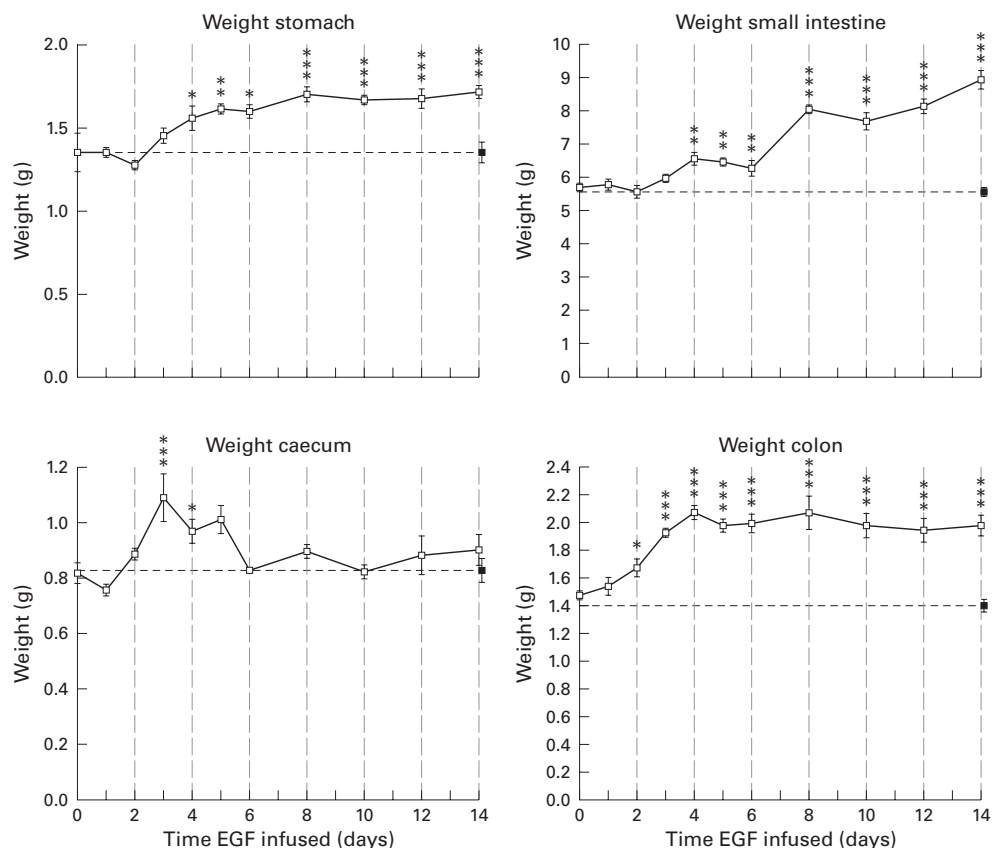
Figure 1 The weights of the major gastrointestinal organs. Animals received 60 µg/rat/day epidermal growth factor (EGF) at time 0. Significantly different from day 14 saline infused controls (Dunnet's test): *p<0.05, **p<0.01, ***p<0.001.

EGF per rat per day. Four rats were killed after 1, 2, 3, 4, 5, 6, 8, 10, 12, and 14 days of infusion. Control rats were also killed at the start and end of the experiment (four on day 0 and six after 14 days of saline infusion).

At the end of the study, animals were killed two hours after injection of 1 mg/kg of vincristine (David Bull Laboratories, Harris Road, Warwick). The wet weights of the various parts of the gastrointestinal tract were recorded and samples of the small intestine and colon (defined by their percentage length) were fixed in Carnoy's fluid and stored in 70% (v/v) ethanol.

All animal procedures were approved by the animal ethics committee of the Imperial Cancer Research Fund and conformed to the regulations of the Animals (Scientific Procedures) Act 1986

Pieces of fixed tissue (from the proximal small intestine and mid colon) were hydrated, hydrolysed, stained with the Feulgen reaction, and then transferred to 45% acetic acid and the crypts teased apart under a dissecting microscope (×40). Crypts were transferred to a glass microscope slide, flattened gently beneath a coverslip, and examined under a compound microscope. The number of blocked metaphases per crypt was counted in 20 crypts per animal,[5] and 200 crypts were scored to determine the crypt branching index.

Further subsamples of tissue were used to determine crypt and villus area, which were determined by tracing the crypts using a drawing tube attached to the microscope.[5] The scale of these was set using a calibrated stage micrometer slide. The drawings were then scanned with a flatbed scanner connected to an Apple Macintosh computer, and the area was measured using the program Image (NIH Public Domain).

STATISTICS

All results are presented as group mean (SEM). Data were tested by analyses of variance. Where statistical significance of ANOVA was reached (p<0.05), individual comparisons against (day 14) control values were performed using Dunnet's test, a method equivalent to repeated measures analyses. In addition, the non-parametric two sided runs test was used to determine if the data were non-random.

Results

WET WEIGHTS OF ORGANS

EGF caused progressive increases in the weights of the stomach, small intestine, and colon but caused only a transient increase in the weight of the caecum (fig 1). At the end of the 14 day infusion period, the weights of the stomach and colon appeared to have reached plateau levels although the small intestine did not appear to have reached its maximal level (fig 1). No effect of EGF was seen on the weight of the pancreas (data not shown).
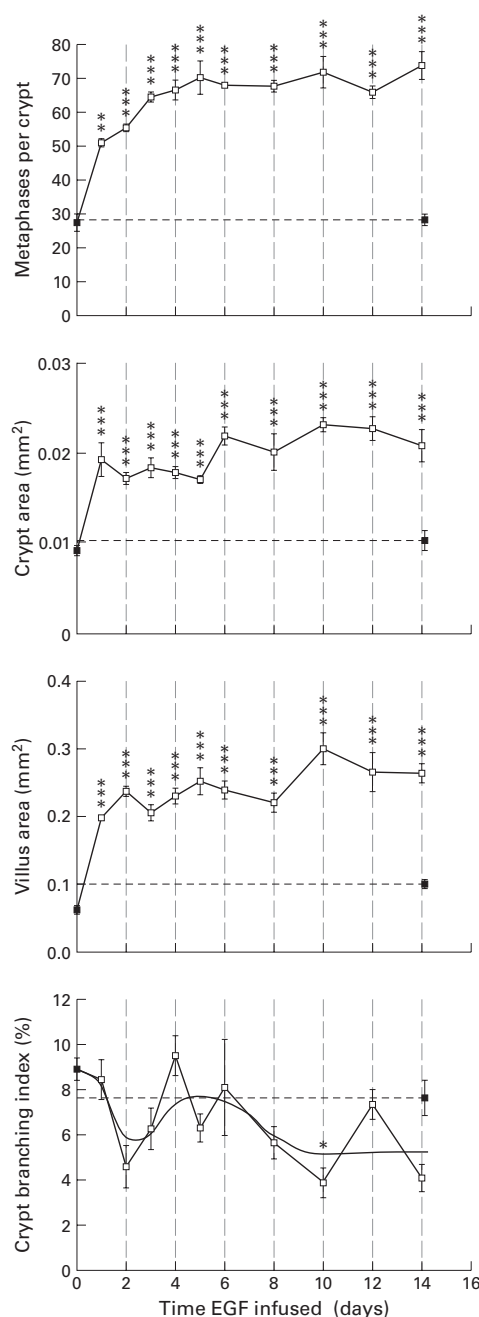
Figure 2   Effect of epidermal growth factor (EGF) on morphology and proliferation in the proximal bowel (10% of the length of the small intestine). Animals received 60 μg/rat/day of EGF at time 0. Significantly different from day 14 saline infused controls: *p<0.05, **p<0.01, and ***p<0.001. All of these parameters were significant by analysis of variance (p<0.001) and the runs test (p<0.001, 0.0026, and <0.001, respectively). The actions of EGF on crypt branching were less pronounced, and indicated a more gradual decrease in crypt branching; nevertheless, analysis of variance was significant at p<0.001 and the runs test also showed an effect at p=0.023.
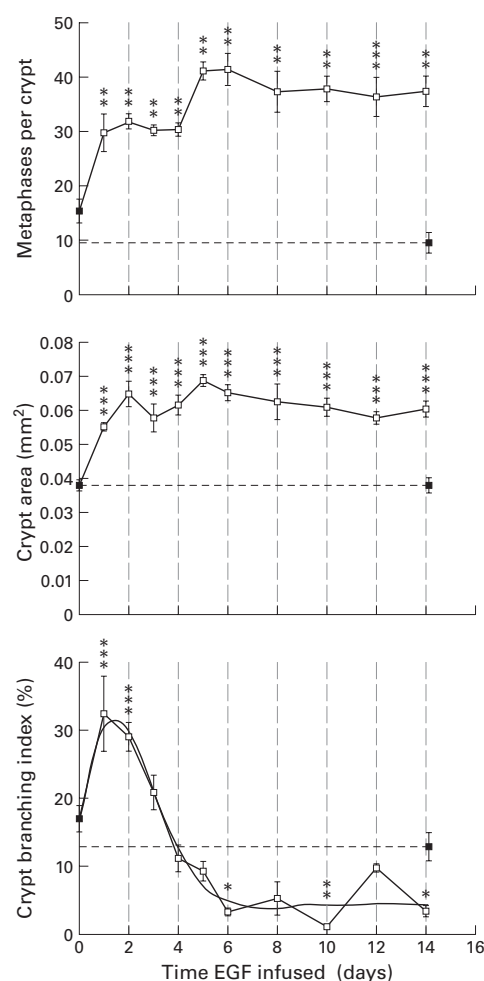

Figure 3   Effect of epidermal growth factor (EGF) on morphology and proliferation in the mid colon (50% of the length of the colon). Animals received 60 μg/rat/day of EGF at time 0. Significantly different from day 14 saline infused controls: *p<0.05, **p<0.01, ***p<0.001. Mitotic activity and crypt area were both significantly greater by analysis of variance (p<0.001) and by the runs test (p=0.03 and 0.047, respectively). Changes in crypt branching were significant at p<0.001 by analysis of variance and 0.0002 by the runs test.

MORPHOMETRIC ASSESSMENT OF THE SMALL INTESTINE

The effects of EGF on the morphometric parameters of the proximal small intestine are presented in fig 2. The number of arrested metaphases per crypt was about twice that of control animals after one day of EGF infusion and values continued to increase until plateau levels were reached by day 5, achieving values which were about 150% greater than the starting level. Crypt and villus areas were also twice those of control animals following one day of infusion of EGF but, in contrast with results from metaphase assessment, there was little further increases seen after infusion of EGF for longer periods. Animals which had received EGF for 14 days had 160% higher metaphase counts per crypt, 100% greater crypt areas, and 180% greater villus areas (all p<0.01). The actions of EGF on crypt branching were less pronounced and showed a more gradual decrease in crypt branching (p=0.023 by the runs test).

MORPHOMETRIC ASSESSMENT OF THE COLON

The number of metaphase counts per crypt increased by 93% after one day of EGF infusion and continued to rise until reaching plateau levels at about day 5 (fig 3). Animals who had received EGF for 14 days had 280% more metaphases per crypt compared with animals not receiving EGF. Crypt area after EGF showed a similar pattern to that seen for

the number of metaphases. Animals that had received EGF for one day had crypt areas of about 145% of controls with values of about 160% being reached in animals receiving EGF for 14 days (all p<0.01). Crypt branching increased by about twofold in animals who received EGF for one or two days; longer periods of infusion resulted in a marked decrease (27% of that of saline infused) in the number of branched crypts (fig 3).

Discussion

We have shown that EGF is a potent stimulus for growth of the gastrointestinal tract, increasing the wet weight and number of arrested metaphases per crypt. The response of crypt branching to EGF was more complicated, showing peaks and troughs depending on the time point studied. The proliferative response to EGF was extremely rapid with most of the increase in wet weight and villus area being achieved within the first two days of infusion. This again demonstrates that the gastrointestinal cell renewal system is very dynamic. The actions of EGF on cell proliferation were most pronounced in the colon[6 7] with effects on crypt branching even more pronounced. Other growth factors, such as keratinocyte growth factor[8] or glucagon-like peptide II,[9] are more potent in the proximal regions of the gastrointestinal tract.

Although a rise in proliferative rates increases the number of cells within a crypt, it does not facilitate replacement of lost crypts. There is therefore currently much interest in the mechanisms by which new crypts are produced in adults. Crypt production consists of a founder crypt becoming branched, which subsequently separates into two or more daughter crypts. This mechanism of new crypt production is called crypt fission, with the branching stage being the visible stage of the process.[10] Crypt fission is now thought to be of major importance in intestinal development[11] and is also increased following intestinal damage and ulceration.[12 13] Fission is also increased in rats given chemical carcinogens[3] and in mice and humans with precancerous defects.[14] It is thus likely that crypt fission may provide a mechanism for the duplication and fixation of stem cell mutations and may thus contribute to the spread of mutated crypts.[15 16]

The present study showed that EGF caused a marked rise in the number of branched crypts within the colon for the first two days of EGF infusion, which was followed by a fall in the number of branched crypts. While the reduction in crypt fission in the colon after one week of infusion confirms our previous results,[17] the earlier increase in fission was not expected as our previous study of a neonate with necrotising enteritis indicated that there was an initial reduction in fission which preceded increased proliferation.[18] One possible explanation for this is that EGF acted as a stimulant for crypt fission/branching and then reduced the number of "resting" branched crypts present in the mucosa. Some of the branched crypts seen under normal circumstances may represent a resting stage, which acts as a reservoir for new crypt production when a suitable stimulus occurs. A large number of branched crypts are seen in the mid colon of adult germ free rats confirming that crypts can indeed rest in the branched state.[19] An alternative explanation is that EGF acted as a stimulant for fission but then exhibited tachyphylaxis (the rapidly decreasing response to an agent following administration of the initial dose). The data obtained from the small intestine did not show this initial increase in crypt branching. The small intestine has a more rapid rate of proliferation than the colon and there would appear to be major differences in the crypt branching response in the two regions of the bowel. The higher incidence of crypts in fission in the colon compared with the small intestine may be of great significance; this difference is most pronounced in the young (rat) and the greater fission incidences in the colon can lead to a more rapid evolution of wholly mutated crypts in the colon following mutagen administration.[15] The colon also may have fewer stem cells,[20] which may augment this. In addition, the results of this study show that the colon is also more responsive than the small intestine (in terms of crypt fission), and although crypt fission is part of the adaptive repertoire, increased fission can also be a risk factor for carcinogenesis.

Crypt fission is likely to be of particular importance for the elusive intestinal stem cell as intestinal proliferation is a hierarchical process in which a small number of cells near the base of the crypt either have innately different characteristics or are in a unique "niche". These cells can give rise to all of the intestinal cell types[21] and are the functional intestinal stem cells[22] and as such are likely to be the main target for carcinogenesis inasmuch as they do not migrate whereas their daughter cells are ephemeral.

There is currently uncertainty regarding the relationship between proliferation and crypt fission; Totafurno *et al* suggested that the two process are linked, with proliferation increasing crypt volume which in turn acts as the stimulus for fission.[10 23] The finding that colonic crypt volume was high when the number of branched crypts seen was low suggests that crypt volume may not be the only factor involved.

It has been suggested that crypt reproduction by fission may be a compensatory response to crypt loss.[17] Potten[24] observed that apoptosis in the proliferating zone of crypts is induced by cytotoxic chemicals. If apoptosis occurs in the stem cells, stem cell loss may lead to crypt deletion. Moreover, the non-polypoid mucosa of familial adenomatous polyposis (FAP), colon cancer patients, and that of the murine homologue of FAP, namely the multiple intestinal neoplasia (*Min*) mouse,[25] have elevated rates of crypt fission.[26] This lends weight to the hypothesis that altered crypt fission may play an important role in the process of carcinogenesis. The development of aberrant crypt foci (ACF) in rats treated with DMH may also be due to a fission mechanism where indentations appear at the base of a single ACF crypt, with subsequent formation of multicryptal ACF,[27] and the size of ACF (multicrypt aberrant crypt multiplicity)

has been shown to be a better predictor of subsequent tumorigenicity than the number of ACF.[28][29] Crypt fission may thus be the means by which mutated clones spread through tissues before becoming overtly invasive. While there is substantial evidence in favour of independent origins of each tumour from a unique mutated clone, there are instances where such clones expand and remain cohesive, often involving a large area of tissue.[16]

In conclusion, we have shown that crypt fission as well as cell proliferation can play a major role in colonic adaptation, so that the gut can respond to growth signals in a variety of ways. The implications of this have yet to be determined but some of the findings of increased fission rates in carcinogen treated animals and humans with FAP would suggest that increased crypt fission, perhaps related to decreased cell-cell adhesion, is a major factor in the progression of cancer.

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